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Spatial and temporal regulation of the homeotic selector gene *Antennapedia* is required for the establishment of leg identity in *Drosophila*

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Abstract

Antennapedia is one of the homeotic selector genes required for specification of segment identity in *Drosophila*. Dominant mutations that ectopically express *Antennapedia* cause transformation of antenna to leg. Loss-of-function mutations cause partial transformation of leg to antenna. Here we examine the role of *Antennapedia* in the establishment of leg identity in light of recent advances in our understanding of antennal development. In *Antennapedia* mutant clones in the leg disc, Homothorax and Distal-less are coexpressed and act via *spineless* to transform proximal femur to antenna. *Antennapedia* is negatively regulated during leg development by *Distal-less*, *spineless*, and *dachshund* and this reduced Antennapedia expression is needed for the proper development of distal leg elements. These findings suggest that the temporal and spatial regulation of the homeotic selector gene *Antennapedia* in the leg disc is necessary for normal leg development in *Drosophila*.

Keywords: *Drosophila*; Leg development; *Antp*; *Dll*; *hth*; *ss*; *dac*

Introduction

The adult body of *Drosophila* possess specialized structures such as leg and antenna to perform specific functions. These adult appendages derive from specialized precursor structures called imaginal discs, which are specified during embryogenesis and acquire segment-specific identity through the action of the homeotic selector genes of the Antennapedia and Bithorax complexes (reviewed in Cohen, 1993). Hox genes are expressed in the primordia of the imaginal discs in the post-oral head (gnathal segments), thorax, and abdomen region. The eye–antenna imaginal disc, the precursor for the adult eye and the antenna, originate in the cephalic region of the embryo, outside the domain of Hox gene expression in the embryonic ectoderm. Thus, the antenna develops without the input of Hox gene expression. Antenna has been suggested to represent the ground state for limb development that arises in the absence of the HOX genes (Struhl, 1981). Mutants in the flour beetle *Tribolium* that remove the entire HOX complex develop antenna-like appendages in every segment instead of leg appendages favoring further that antenna may represent the ground state for limb development (Beeman, 1987; Stuart et al., 1991).

Leg and antenna have long been considered to be homologous structures by morphology (Snodgrass, 1935). The homeotic gene *Antennapedia* (*Antp*) of the Antennapedia complex has been proposed to be the key selector gene responsible for leg identity (Gehring, 1966; Struhl, 1981). As expected of a selector gene, *Antp* is expressed in the leg but is not expressed in the antennal disc (Casares and Mann, 1998). Dominant mutants in the *Antp* gene where there is ectopic expression of Antp in the antenna disc cause transformation of antenna to leg provided further genetic support for this view (Gehring, 1966; Postlethwait and Schneiderman, 1971). Ectopic expression of *Antp* or *Sex combs reduced* genetic modifications can also cause transformation of antenna toward T2 or T1 leg, respectively (Gibson and Gehring, 1988; Zeng et al., 1993). *Antp* is expressed in leg discs and has been proposed to repress expression of genes required for antenna identity (Casares and Mann, 1998; see Fig. 1).

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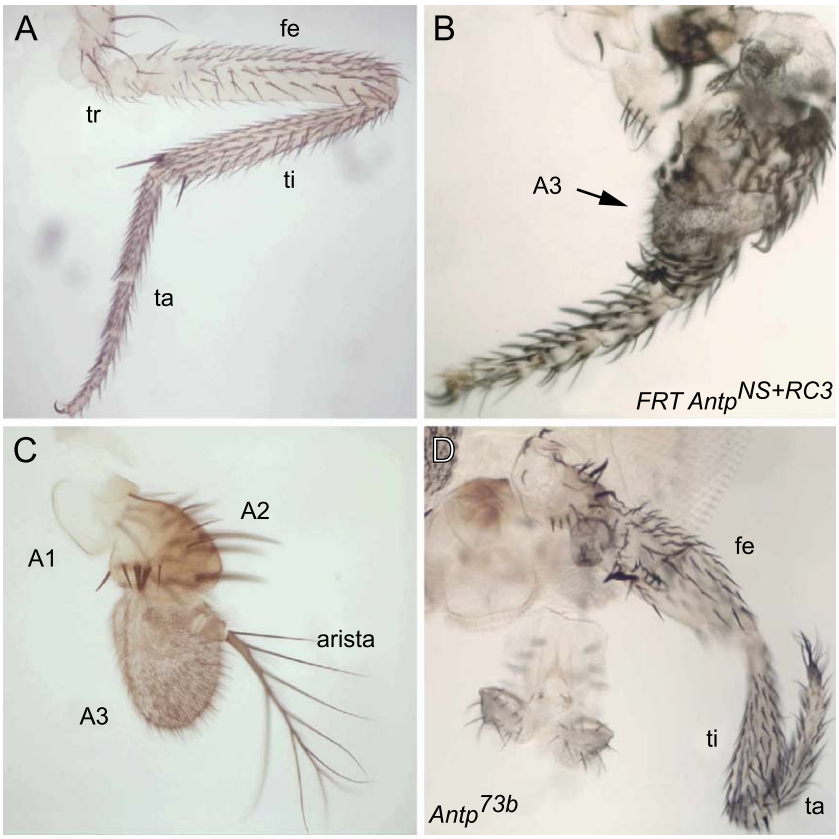

Fig. 1. *Antp* phenotypes. (A) Cuticle preparation of a wild-type second thoracic leg. Abbreviations: tr, trochanter; fe, femur; ti, tibia; ta, tarsal segments. (B) Cuticle preparation of a second thoracic leg with *Antp* null mutant clones. Mutant cells were genetically marked with *forked.* Arrow indicates antennal tissue in the femur. (C) Cuticle preparation of a wild-type antenna. Abbreviations: A1–A3, first to third antennal segments. (D) Cuticle preparation of the head of an $Antp^{73b}$ mutant fly. Antenna is extensively transformed to leg.

The patterning mechanisms that organize the anteroposterior, dorsoventral, and proximodistal axes of the leg and antenna are comparable. During the normal course of limb development, it was suggested that Hedgehog signals from the posterior cells to induce Wingless (Wg) and Decapentaplegic (Dpp) expression in anterior cells (Basler and Struhl, 1994). Wg and Dpp specify ventral and dorsal fates, respectively (Diaz-Benjumea et al., 1994; Struhl and Basler, 1993) and repress each other's expression to maintain dorsoventral axial patterning (Brook and Cohen, 1996; Heslip et al., 1997; Jiang and Struhl, 1996). Concurrently, the combined action of Wg and Dpp organizes the proximodistal axis of the leg and antenna by regulating the expression of Distal-less (Dll), Dachshund (Dac), Homothorax (Hth), and Teashirt (Tsh) in discrete domains along the proximodistal axis (Abu-Shaar and Mann, 1998; Dong et al., 2001, 2002; Lecuit and Cohen, 1997; Wu and Cohen, 1999, 2000).

Although the underlying patterning mechanisms are the same, the expression of some of the proximodistal axial target genes of Wg and Dpp differs in leg and antenna. Antennal identity depends on overlapping expression of the transcription factors *hth* and *Dll* (Casares and Mann, 1998; Dong et al., 2000; 2002). *hth* encodes a TALE class homeodomain protein that promotes nuclear localization of Extradenticle, which also serves as cofactor for homeodomain proteins (Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997; Ryoo et al., 1999). *Dll* encodes a homeodomain protein required for the normal development and differentiation of all distal segments of the limbs (Cohen and Jürgens, 1989; Cohen et al., 1989; Gorfinkiel et al., 1997). zOverlapping expression of Hth and Dll controls the antenna-specific expression of other genes including *cut* and *spineless* (*ss*) (Duncan et al., 1998; Emerald et al., 2003). *spineless* encodes a basic-helix-loop-helix-PAS domain containing transcription factor, related to the mammalian dioxin receptor. *spineless* is expressed in distal regions of the antenna and leg discs in second instar and is maintained in the antenna but not in the leg (Duncan et al., 1998). *spineless* in turn controls expression of the *distal antenna* (*dan*) and *distal antenna related* (*danr*) genes. *dan* and *danr* encode members of a family of nuclear proteins with a pipsqueak domain that are expressed in the eye–antennal disc and contribute to specification of distal antennal identity (Emerald et al., 2003).

Although it has been shown that the homeotic selector gene *Antp* is the key factor determining leg development,

our understanding of the mechanisms by which Antp establishes leg identity is not complete. It is also an open question how the morphological differences between the different segments along the proximodistal axis of the limb are generated and maintained. As a first step toward understanding how these processes are coordinated, we examine, in this report, the function of the homeotic selector gene *Antp* in leg development in light of the recent advances in our understanding of antenna development. Using lineage tracing of *Antp*-expressing cells, we show that Antp is initially expressed throughout the leg imaginal disc. We also show that the genes *Dll* and *dac* down regulate Antp expression as the discs grow and this down-regulation is needed for the normal development and differentiation of distal leg segments. We also present evidence that at least part of this function of *Dll* in the distal leg is mediated through *ss*. Thus, we suggest that the simple concept that Antp functions as a leg selector gene be modified to include the idea that its expression must be spatially and temporally regulated, indeed removed, to allow normal leg development.

Materials and methods

Drosophila stocks

$ss^{D114.4}$, $ss^{D115.7}$, *UAS-ss* are described in Duncan et al. (1998). *UAS-Dll* is described in Wu and Cohen (1999). ss^{a}, $Antp^{73b}$, $Antp^{NS+RC3}$, *Dll*, *dac* alleles are described in flybase (http://flybase.bio.indiana.edu.82).

Genotypes of larvae used for clonal analysis

Dac: *hsFlp1*; dac^{3}*FRT40A*/M*(2L)25A* arm^{lacz}*(36B) FRT40A*
Dll: *hsFlp1*; *FRT42B* Dll^{SAI}/*FRT42B* arm^{lacZ} *(51D) M(2R)58F*
Antp: *hsFlp f*; *FRT82B* $Antp^{NS+RC3}$/*FRT82B* arm^{lacZ} *(83)*f^{+} *(87D) M(3R)95A*.

Lineage tracing

AntpGal4 was generated by gene conversion. The $Antp^{lacZ}$ (pAPT1.0-84) transgene insertion in the endogenous *Antp* P1 promoter (Engstrom et al., 1992) was used for gene conversion. *MS1096Gal4;* $Antp^{lacZ}$*/Δ2-3TMS* male flies were crossed to *w/w; TM6B/TM3Sb* females. Male progeny carry the *w* mutation on their X chromosome. Males with w^{+} eyes may have the desired gene conversion event. Stocks were established and tested for GAL4 expression using *UAS-EGFP*. Two independent lines, *AntpGal4 10* and *AntpGal4 21*, were established. Line 21 is used for the experiments described here. Estrogen-regulated Flip recombinase (*UAS-Flp-EBD*) was used as described (Weigmann and Cohen, 1999).

Antibodies

The following antibodies were used: Rat anti-Dll (Wu and Cohen, 2000), mouse anti-Dll (Duncan et al., 1998), mouse anti-Antp (Condie et al., 1991), rat anti-Antp (provided by Maria Capovilla), rabbit anti-βgal (Cappel), rat anti-Dan, mouse anti-Dan (Emerald et al., 2003).

Results

Previous studies have reported partial transformation of leg to antenna in clones lacking the function of *Antp* (Casares and Mann, 1998; Struhl, 1981). To examine how *Antp* specifies leg identity, we have made use of the FLP-FRT system (Xu and Rubin, 1993) to generate clones mutant for the null allele $Antp^{NS+RC3}$ (Struhl, 1981). Analysis of the transformations caused by these clones is facilitated because clones can be induced at high frequency and can contribute to both A and P compartments. In addition, clones can be large because of the growth advantage provided by the Minute technique (Morata and Ripoll, 1975). As reported previously, $Antp^{NS+RC3}$ clones only caused transformation in part of the leg (Casares and Mann, 1998; Struhl, 1981). In most cases, femur was transformed to third antennal segment (Fig. 1). Mutant cells in more proximal and more distal leg segments produced well-differentiated leg tissue, although the morphology of the leg was severely perturbed (see also Fig. 7A). One explanation for the localized transformation seen in the absence of Antp may be the stability of Antp protein. If the protein is stable, we may possibly not be able to see the mutant phenotype even though the tissue is mutant, as suggested previously (Casares and Mann, 1998; Struhl, 1981, 1982). This explanation assumes differential stability or differential dilution by growth in femur and other leg segments. To explore this possibility, we have examined discs from the dominant gain of function mutation of *Antp*, $Antp^{73b}$ where there is ectopic expression of Antp in the antenna disc resulting in antenna to leg transformation (Gehring, 1966; Fig. 1D). We noted that the distal-most segments of the transformed antenna discs were devoid of Antp expression, suggesting the distal-most segments of the transformed leg tissue may develop without input from Antp. Thus, we consider differential stability of Antp in proximal and distal leg regions as an unlikely explanation for the specific transformation seen in the femur but not in more distal leg segments.

Regulation of Antp expression in the leg disc

During the embryonic stages of development, Antp is shown to express in all the leg primordia, favoring its role as a leg-specific selector gene (Casares and Mann, 1998). But the domain of *Antp* expression in the mature leg disc does not allow an easy explanation for the localized focus of transformation seen in the femur. Antp is expressed in a ring

in the proximal part of the leg disc and a much lower level in more distal leg regions (Fig. 2A; see also Casares and Mann, 1998; Wirz et al., 1986). The domain of strong *Antp* expression overlaps Hth in the presumptive body wall, coxa, and trochanter segments and extends a few cell diameters distal to the Hth domain (Fig. 2B), where it overlaps with Dac expression in the presumptive proximal femur (arrow, Fig. 2C). We have generated null clones of *dac* and stained them for Antp expression. Interestingly, clones of cells lacking Dac activity show elevated expression of Antp in some cells (Fig. 2D), suggesting that Dac contributes to reducing Antp expression.

Antibody staining has shown that Antp is broadly expressed in the embryonic leg disc primordia and becomes restricted to a more proximal domain as the disc matures (Casares and Mann, 1998). To understand how the developmental changes in this early expression relate to proximodistal regionalization of the leg disc, we have made use of an $Antp^{Gal4}$ line and estradiol-regulated *UAS-FLP* recombinase to lineage tag cells that express Antp at different stages of leg development (*AntpGal4*, *UAS-Flp-EBD act5c lacZ* larvae). In the absence of estradiol, clones of lac^{z} expressing cells were recovered infrequently (16/20 discs had no clones; one disc had three clones; two discs had two clones; one disc had one clone; Fig. 3A). Treatment with estradiol beginning in first instar induced clones all along the proximodistal axis (Fig. 3B), confirming that Antp is expressed throughout the disc at this stage. Estradiol treatment beginning in second instar produced many clones in tibia, femur, and proximal segments, but few in tarsus (Fig. 3C). Clones were mainly recovered in proximal region when estradiol treatment was started in early third instar (Fig. 3D). These observations support the idea that Antp is expressed throughout the disc during the early stages and the expression is gradually lost from the distal segments as the leg disc develops. This was further confirmed by labeling discs of different ages with anti-Dll and anti-Antp (Figs. 4A–C). The observation that Antp is expressed throughout the disc in the early stages and is restricted to the proximal portion during the late stages suggested that establishment of leg identity occurs in distinct steps (1) an early uniform expression of Antp in the disc that prevents the establishment of antenna fate and (2) a later down-regulation of Antp by other factors along the proximodistal axis that is required for normal leg development (Fig. 8). Clonal analysis suggests that Antp is required for longer in the femur than in more distal segments.

Dll has been suggested to be one of the genes needed for the normal development and differentiation of the distal appendages and is expressed from a very early stage of ventral disc development (Cohen and Jürgens, 1989a;

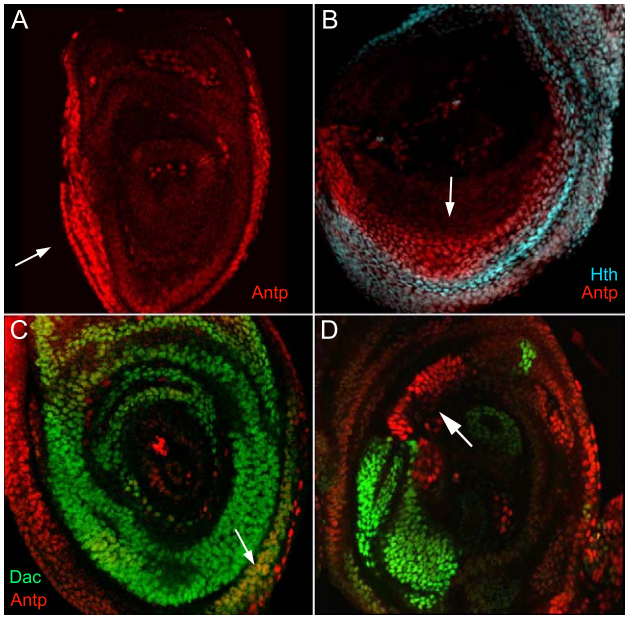

Fig. 2. Antp expression in the leg. (A) Wild-type third instar leg disc labeled with antibody to Antp protein (red). Antp is expressed more strongly in a ring near the periphery of the disc (arrow). (B) Basal optical section of a leg disc labeled to visualize Antp (red) and Homothorax protein (blue). Antp extends distally to Hth (arrow). (C) Overlap of Dachshund protein (green) and Antp in the proximal part of the Dachshund domain (arrow). (D) Elevated expression of Antp in some cells in clone of dac^{3} mutant cells (arrow). Not all cells of the clone showed elevated Antp levels, suggesting that other genes may contribute to repression of Antp.

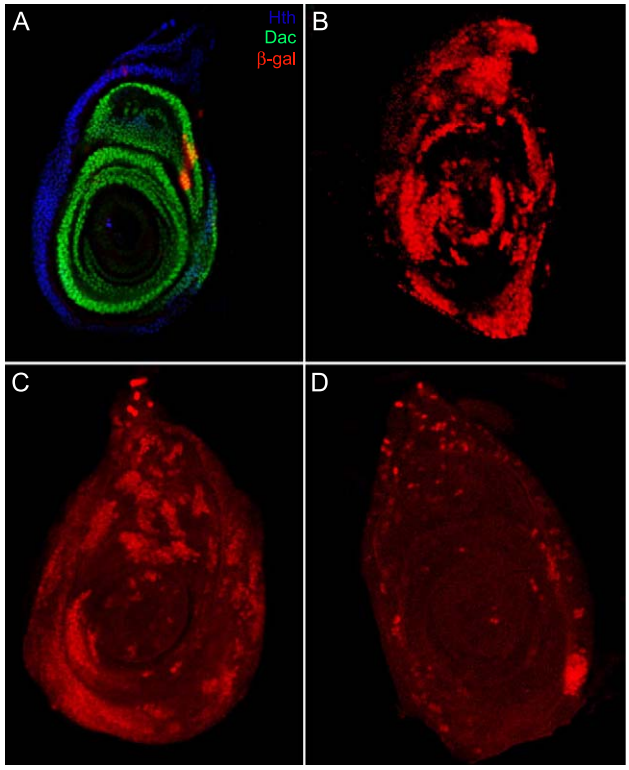

Fig. 3. Lineage tracing of cells expressing Antp at different stages. *AntpGal4* and *UAS-FLP-EBD* were used to drive FLP recombinase in Antp-expressing cells. FLP-EBD activated by addition of water-soluble β-estradiol. (A) Control disc not treated with estradiol and labeled to visualize Hth (blue), Dac (green), and βGal (red). Few clones were observed in control discs, indicating a low basal activity of FLP-EBD. (B–D) Leg discs from larvae induced with estradiol in first, second, and early third instar, respectively. Clones marked by βGal expression (red).

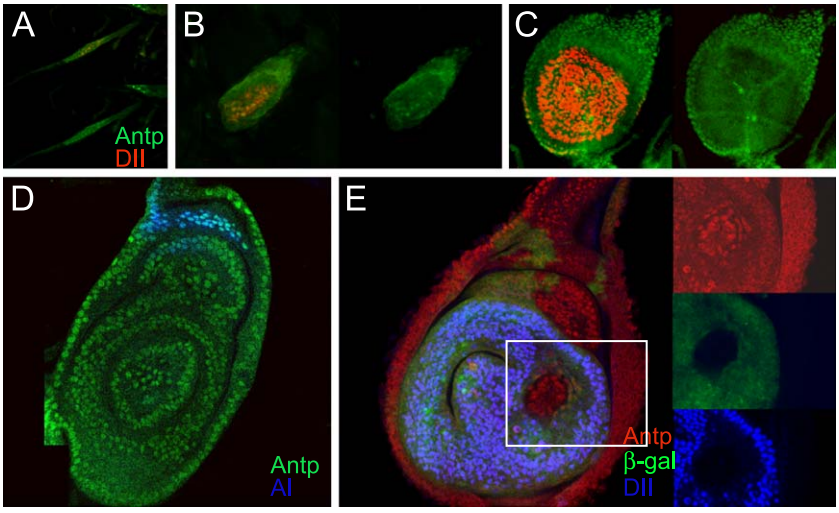

Fig. 4. Antp regulation by Dll. (A–B) Second instar and (C) early third instar leg discs labeled to visualize Antp (green) and Dll (red) proteins. Antp levels are reduced in Dll-expressing cells as the leg develops. (D) *Dll*^{3} homozygous mutant leg disc labeled to visualize Antp (green) and Al (blue). (E) Third instar leg disc with clones of cells mutant for the null allele *Dll*^{SA1}, marked by the absence of βGal (green) and Dll (blue). Antp (red) was up-regulated in the clone. Antp in the boxed regions is shown separately in the inset.

Cohen et al., 1989; Gorfinkiel et al., 1997). To verify the possibility that Dll contributes to the repression of Antp, we examined Antp expression in *Dll* mutants. Antp was expressed at an elevated level throughout the distal region of the *Dll*^{3} homozygous mutant discs (Fig. 4D; discs were genotyped by loss of Aristaless expression in the tarsus). In

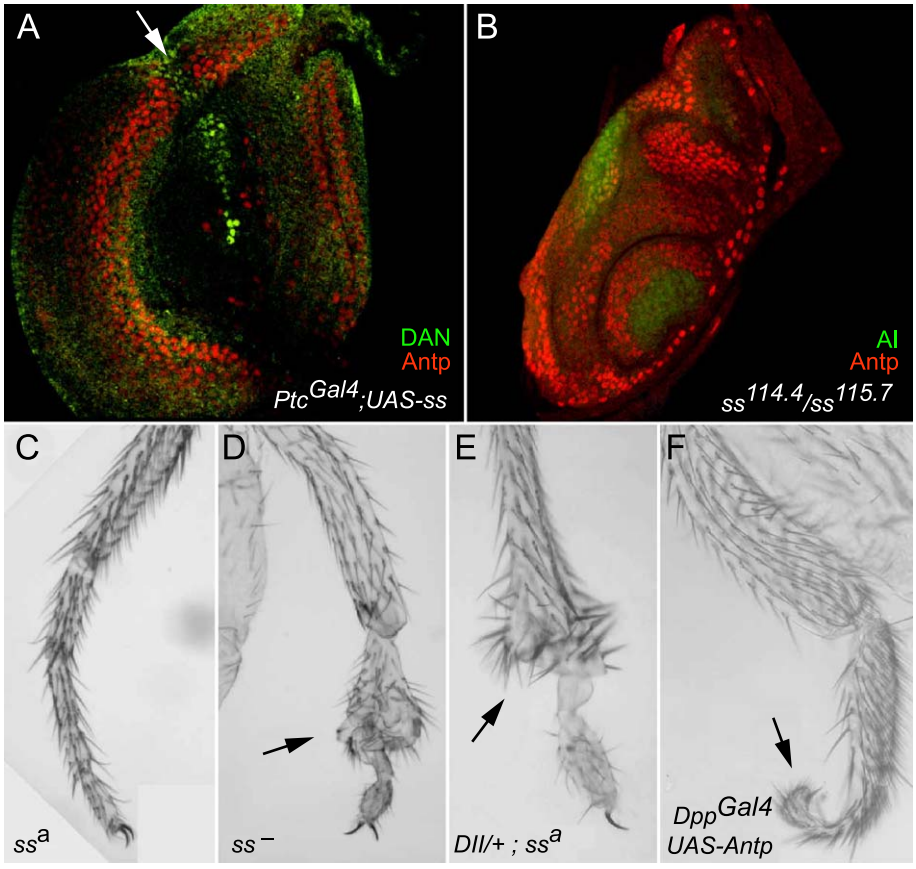

Fig. 5. Antp regulation by *spineless*. (A) Expression of *spineless* under *ptcGAL4* control repressed Antp (red) in the proximal leg disc. Dan protein (green) marks cells expressing *spineless*. (B) Elevated expression of Antp in the distal segments of a *spineless* mutant leg. (C–F) Cuticle preparations of (C) a homozygous *ss*^{a} mutant leg. (D) *Spineless* null mutant combination *ss*$^{D114.4}$/*ss*$^{115.7}$. (E) A homozygous *ss*^{a} mutant carrying one copy of the *DllGal4* allele. (F) Cuticle preparation of a leg expressing Antp under *dppGal4* control. Arrows indicate tarsal fusions.

addition, Antp levels were elevated in Dll^{SA1} mutant clones (Fig. 4E). Antp levels were reduced in Dll-expressing cells in second and early third instar discs (Figs. 4A–C). We also observed that ectopic Dll in the proximal Antp domain down-regulates Antp (not shown). As noted above, clones of cells lacking Dac also showed elevated expression of Antp in the presumptive femur (Fig. 2D), indicating that Dac and Dll both contribute to repression of Antp expression in distinct domains along the proximodistal axis of the leg.

Regulation of Antp by Dll is mediated through spineless

Earlier studies suggested that *ss* is the gene responsible for antennal identity and that it does so by mediating the instructive effects of Hth and Dll in the distal antenna (Dong et al., 2002; Duncan et al., 1998). *ss* shows a dynamic pattern of expression in the leg. It is transiently expressed in the distal leg during second instar and this transient expression is needed for the tarsal development (Duncan et al., 1998). Because *ss* is a target of Dll in the antenna *ss* is possibly responsible for Dll-mediated repression of Antp. To test this, we ectopically expressed *ss* using *ptcGal4*. Antp expression was repressed where *ss* overlapped its proximal expression ring (Fig. 5A; *ss* activity was visualized by expression of its target gene Dan, Emerald et al., 2003). Dan is a downstream target of *ss* and is specific for the antenna. It is ectopically expressed in the leg when Ss is ectopically expressed and its expression is absent in the antenna when *ss* expression is lost. Dan can also be ectopically expressed in the leg when the upstream regulators of *ss*, *Dll*, or *hth* is expressed ectopically in the endogenous domain of the other protein (Emerald et al., 2003).

We next examined discs from the null mutant combination of $ss^{114.4}/ss^{115.7}$ for Antp expression. Absence of normal expression of *ss* results in fusion of tarsal segments in the leg (Fig. 5D). In those discs, Antp was expressed at elevated levels in most of the distal part of the leg disc, suggesting further that *ss* contributes to the reduction of Antp expression in the leg disc (Fig. 5B). In a weaker mutant allele of *ss*, ss^{a} where tarsal defects were not normally observed, removing one copy of the *Dll* gene caused tarsal defects (Figs. 5C,E). This is consistent with the possibility that Dll regulates Ss to repress Antp expression. Ectopic expression of Antp in the tarsal region under *dppGal4* control was sufficient to cause fusion and distor-

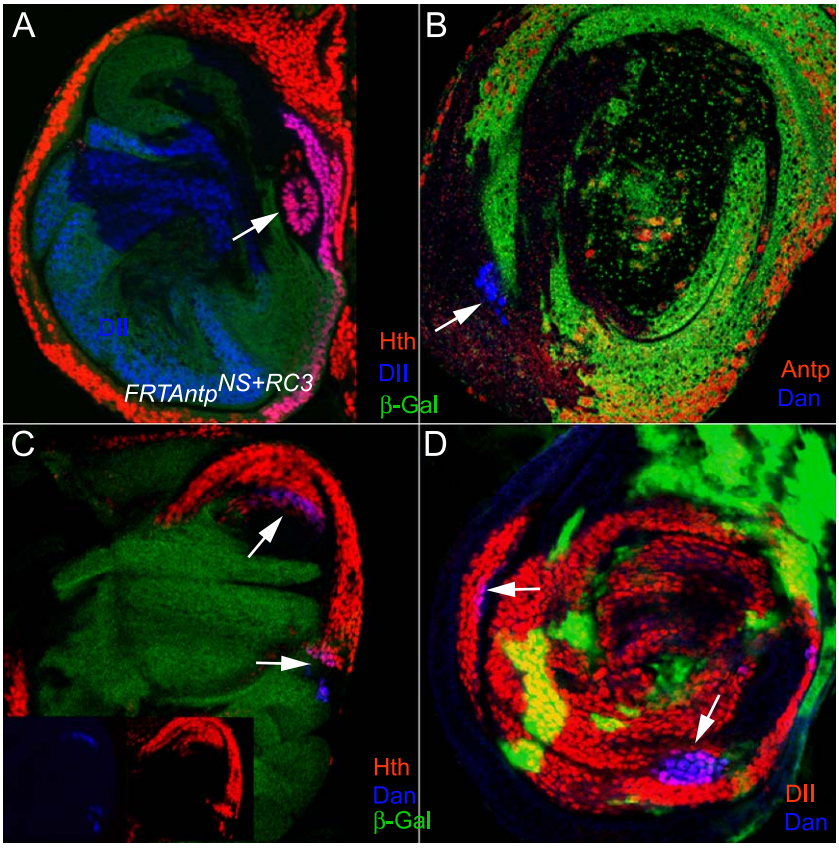

Fig. 6. Antenna marker gene expression in *Antp* mutant clones in the leg. *Antp* mutant clones were marked by the absence of βGal protein (green). (A) Overlapping expression of Hth (red) and Dll (blue) in an Antp mutant clone (arrow). Abnormal expression is localized and causes alterations in the folding of the disc. There was no effect in the endogenous Hth or Dll domains. (B) Expression of the antenna-specific protein Dan (blue) in an Antp mutant clone (arrow). (C, D) Dan ectopic expression occurred in close conjunction with Hth- or Dll-expressing cells in Antp mutant clones (blue, arrows). Coexpression of ectopic Dan (blue) and Hth (red) is shown in the insert in C.

tion of tarsal segments (Fig. 5F). Together, these observations indicate that repression of Antp in the distal leg is necessary to allow normal development. We note that the distal-most tarsal elements are unaffected in the *ss* mutant and that Antp is not expressed there. This coincides with the domain in which *al* is expressed (green, Fig. 5B), suggesting that an additional repressor of *Antp*, perhaps *al*, is also required in the tip of the leg disc.

A focus for transformation in the presumptive femur

To better understand the basis for the spatially limited transformation in *Antp* mutant clones, we have examined the expression of genes implicated in antenna development. A fundamental difference between leg and antenna development is observed in the control of Hth and Dll expression. Hth is expressed in the three proximal segments in the antenna and Dll is expressed from the second antennal segment to the distal end. Thus, they overlap extensively in the second and third antennal segments, but only to a very limited extent in the leg disc (Casares and Mann, 1998; Dong et al., 2000; Wu and Cohen, 1999). We have generated $Antp^{NS+RC3}$ mutant clones and assessed whether there is coexpression of Hth and Dll as in the second and third antennal segment. Overlapping expression of Hth and Dll was observed in clones in the presumptive femur region of the disc (Fig. 6A). This overlap was localized and did not occur in all cells lacking Antp. Ectopic Dll did not occur in Hth-expressing cells of the coxa. Likewise, ectopic Hth did not occur in Dll-expressing cells of the tibia or tarsus. Thus, there appears to be a specific focus for transformation in the proximal femur that corresponds well to the observed transformation in adult tissue. In this context, it is important to note that Hth and Dll can transform more proximal and more distal leg elements to antenna when the two proteins are coexpressed using the Gal4 system (Dong et al., 2000).

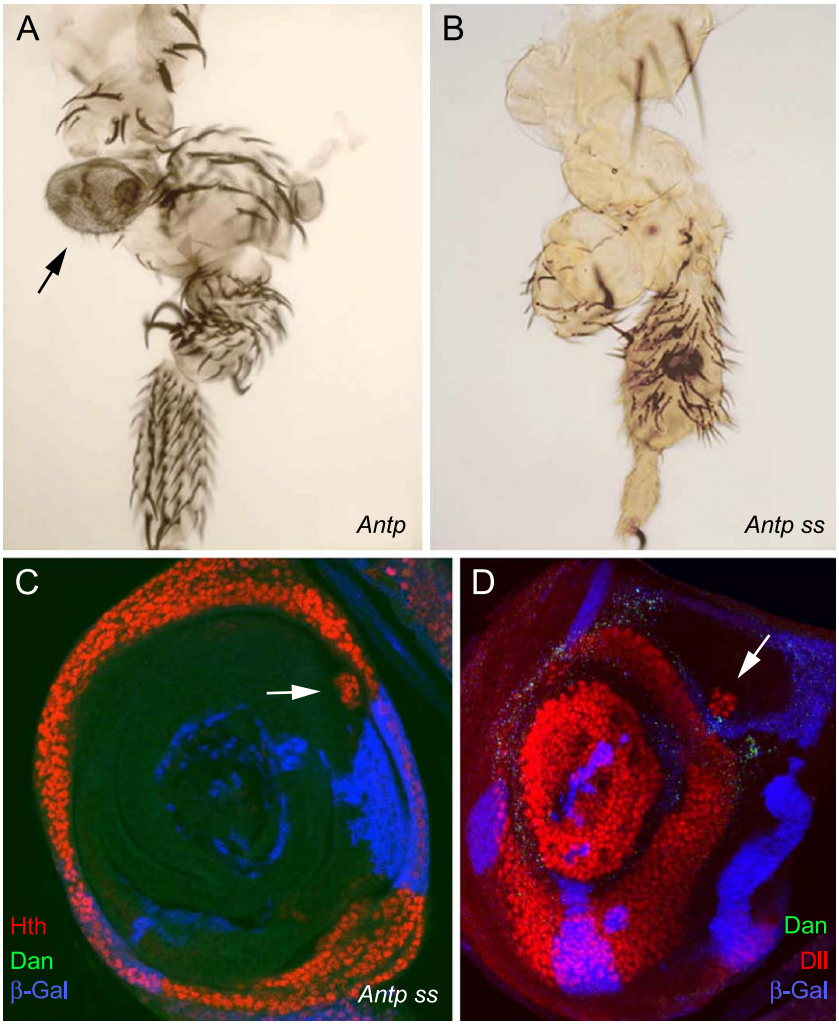

Fig. 7. *Spineless* is required for transformation from leg to antenna in *Antp* mutant clones. (A) Cuticle preparation of a second leg with large $Antp^{NS+RC3}$ clones marked with *forked*. Mutant cells differentiate as third antennal segment in the femur (arrow). (B) Cuticle preparation of a second leg with large $Antp^{NS+RC3}ss^{115.7}$ double-mutant clones, marked with *forked*. All mutant cells had leg identity. (C, D) Leg discs with multiple $Antp^{NS+RC3}ss^{115.7}$ mutant clones marked by the absence of βGal (blue). Ectopic Hth or Dll expression (arrows) was not associated with Dan expression.

In the antenna, Dll and Hth act through *ss* to induce expression of the antenna-specific gene *dan* in the distal antennal segments (Emerald et al., 2003). $Antp^{NS+RC3}$ mutant clones showed localized ectopic expression of the antenna-specific protein Dan (Emerald et al., 2003; Fig. 6B). Ectopic Dan expression was only observed on the proximal region of the leg disc overlapping with Hth- or Dll-expressing cells (Figs. 6C,D). Thus, the pattern of ectopic Dan expression in the leg reflects the combined activity of Hth and Dll and suggests that the localized transformation seen in the Antp follows the normal mode of regulation in the antenna disc (Emerald et al., 2003).

Antp mutants act via spineless

Coexpression of Hth and Dll in the antenna leads to stable expression of *ss* in a domain corresponding to the third antennal segment and the arista (Dong et al., 2002; Duncan et al., 1998). *ss* is transiently expressed in the distal leg during second instar but is not maintained. Coexpression of Dll and Hth in the *Antp* mutant leg tissue might lead to abnormal maintenance of *ss* expression in the leg disc. Similarly, ectopic expression of *ss* in the leg induces ectopic expression of antenna-specific gene *dan* (Emerald et al., 2003). Antibody to Spineless protein is not available, so we cannot assess its expression in *Antp* clones. As an alternative, we asked whether *ss* activity is required for transformation of leg in *Antp* mutant clones. Clones mutant for *Antp* were compared with clones simultaneously mutant for *Antp* and *ss* (using the null alleles $Antp^{NS+RC3}$ and $ss^{115.7}$). As described above, *Antp* clones caused transformation of femur to A3, without obviously affecting the identity of more proximal or more distal leg segments (Fig. 7A). These clones were associated with other abnormalities including fusion of leg segments. *Antp ss* double-mutant clones caused similar segment fusion defects, but showed no sign of transformation to antenna (Fig. 7B). Interestingly, *Antp ss* double-mutant clones showed limited ectopic expression of Hth and Dll (arrows, Figs. 7C,D), as observed in *Antp* single-mutant clones (Fig. 6A). However, despite coexpression of Hth and Dll, the antenna-specific Dan protein was not expressed in the *Antp ss* double-mutant clones. In the antenna, Dan acts downstream of *ss* and contributes to specification of antennal identity (Emerald et al., 2003). The failure to ectopically express Dan is consistent with the lack of transformation observed in the adult legs. These observations indicated that *ss* is an essential mediator of transformation caused by lack of Antp in the leg disc.

Discussion

Antp acts as a selector gene responsible for specification of leg identity (Gehring, 1966; Struhl, 1981, 1982). Dominant *Antp* mutations can cause nearly complete transformation of antenna to T2 leg. The *Sex combs reduced* (*Scr*), *Antp*, and *Ultrabithorax* genes are expressed in the thoracic segments of the embryo in which the leg imaginal discs originate. *Antp* and *Scr* are not normally expressed in the antenna but can cause transformation of antenna toward leg when expressed ectopically (Gibson and Gehring, 1988; Zeng et al., 1993). Loss-of-function mutations removing Antp, or Scr and Antp can cause the reciprocal transformation of leg toward antenna (Struhl, 1981, 1982). However, these transformations are less complete than those caused by the gain-of-function alleles. In this report, we have examined the regulation and function of Antp in leg development. We suggest that specification of leg identity is accomplished primarily by repression of antennal identity and that antenna may represent the ground state for appendage development. In this regard, a recent study on the evolution of Antp function is particularly interesting (Shiga et al, 2002). Antp from the crustacean *Daphnia* was shown to down-regulate *Dll* in the *Drosophila* embryo although *Drosophila* Antp cannot do so. The molecular differences that allow repression by Ubx, but not Antp, have recently been defined (Gebelein et al., 2002).

Complex regulation of Antp in the leg disc

Antp expression is spatially and temporally regulated in the leg disc and this regulation is needed for the normal development and differentiation of the leg. Antp is initially expressed throughout the early leg primordia in the embryo (Casares and Mann, 1998). Based on expression studies and lineage tracing, we present evidence that in the first instar leg disc, Antp is also expressed throughout the disc and its expression is gradually lost from the presumptive distal region of the leg disc during second and early third instar stages. Distal repression of Antp requires Dll, which encodes a homeodomain protein required for specification of distal leg structures (Cohen et al., 1989). We present evidence that Dll may act in part through the *spineless* gene to repress Antp. Dac is expressed in presumptive femur and tibia (Lecuit and Cohen, 1997; Mardon et al., 1994) and also contributes to repression of Antp in this region. A diagram outlining the regulation and function of Antp in leg and antenna discs is presented in Fig. 8. *spineless* encodes a predicted transcription factor, with homology to the mammalian dioxin receptor (Duncan et al., 1998). *spineless* mutants cause transformation of antenna to leg (Burgess and Duncan, 1990; Struhl, 1982). *spineless* is expressed in the distal segments of the antenna under the control of Hth and Dll, where it acts to specify distal antennal identity (Dong et al., 2002; Duncan et al., 1998). *spineless* is transiently expressed in the distal segments of the leg disc and is required for normal tarsal development. Our findings show that *spineless* acts downstream of Dll to reduce the level of Antp expression in most of the distal leg, including femur, tibia, and much of the tarsus. *spineless* also represses Antp in the antenna disc, as indicated

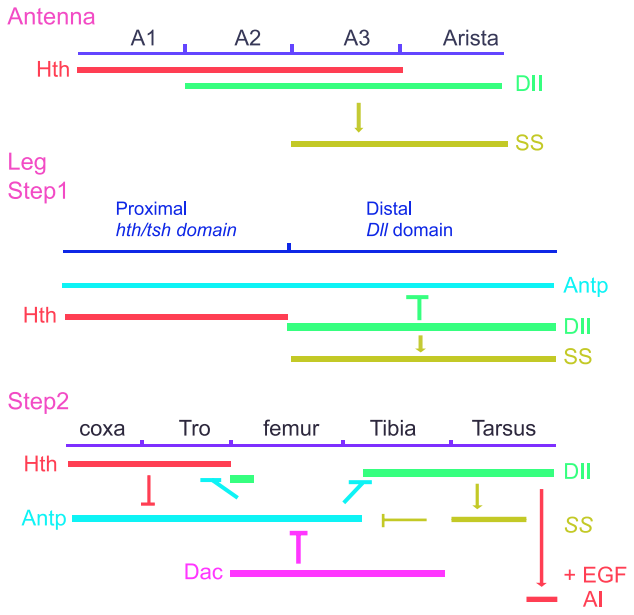

Fig. 8. Regulation and function of Antp in antenna and leg discs (arrows indicate positive regulation and lines with end bars indicate negative regulation). See text for a detailed explanation.

by the observation that transformation to leg in *spineless* mutant antennae is associated with ectopic Antp expression (Emerald et al., 2003).

In the absence of Dll, we observed elevated expression of Antp throughout the distal segments of the leg disc. In the absence of *spineless*, Antp expression was elevated everywhere except in the most distal tarsus where Aristaless is expressed. Recently, it has been shown that the EGF signaling pathway plays an important role in patterning the tarsal elements of the leg (Campbell, 2002; Galindo et al., 2002). Dll has been shown to support tarsal development by promoting *bric-a-brac* expression in distal segments. EGF receptor signaling induced by *vein* acts with Dll to promote the expression of Aristaless in the distal most segment while down-regulating *bric-a-brac* expression. In the *spineless* mutant, Antp is not repressed in this domain. It is not yet clear whether this is a direct effect of Dll plus EGF signaling or if it is mediated through Aristaless.

Antp and leg identity

Ectopic Antp expression can cause quite complete transformations of antenna to leg. Although Antp is expressed throughout the early leg disc, *Antp* mutant clones only caused limited transformation of leg to antenna. Tarsal segments were never affected, even when clones were induced in the blastoderm embryo (Casares and Mann, 1998; Struhl 1981; this work). In the disc, we observed that the alterations in gene expression that cause transformation of leg to antenna only arise at a defined position within the leg disc. Overlapping expression of Hth and Dll was observed only in the proximal femur, close to the endogenous Hth domain. The mechanism by which the femur arises during proximodistal axis formation may provide an explanation for the focus of transformation in *Antp* mutant clones. At early stages of development, the leg disc is subdivided into nonoverlapping proximal and distal primordia, defined by the expression of Dll in the distal zone and Hth/Tsh in the proximal zone (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Wu and Cohen, 1999, 2000). Heterochronic disc transplantation experiments have suggested that only tarsal and body wall primordia are specified at this stage, and that intervening segments arise as development proceeds (Schubiger, 1974). Lineage tracing experiments have confirmed this view and shown that cells giving rise to the femur and tibia region mainly originate in the Tsh/Hth expression domain (Weigmann and Cohen, 1999). Cells in tibia and tarsal leg segments can also derive from the proximal primordium, but cells born in the early Dll domain almost never give rise to femur under normal circumstances (although Dll activity is required at these stages for formation of the femur; see Cohen and Jürgens, 1989). This indicates that there is considerable net movement of cells from the Hth- and Tsh-expressing proximal domain to populate the femur and to a lesser extent more distal segments. Under normal circumstances, these cells of proximal origin must lose Hth expression and acquire Dll expression to contribute to distal segments.

We note that transformation to antenna occurs mainly in the proximal femur and that it is associated with ectopic expression of Hth and Dll. We propose that Antp is required to prevent coexpression of Hth and Dll in the leg. Later, this may be maintained partially by the expression of *dac* in the intermediate domain (Dong et al., 2001). In the absence of Antp, cells that originate proximally might be able to maintain Hth expression while acquiring Dll expression under the influence of Wg and Dpp signals (Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997). According to this view, transformation might be expected to occur in clones that originate proximally. As proximal cells mainly contribute to femur, this may explain the observation that transformation of more distal segments is observed rarely, and that only large clones cause transformation. The observation that *Antp* mutant clones do not affect distal leg elements is difficult to explain in terms of the selector gene model. In fact, our results indicate that repression of Antp is needed to allow normal development of the tarsal segments. This seems surprising given that Antp expression can cause complete distal transformation of the Antenna.

Perhaps ectopic Antp prevents coexpression of Hth and Dll in the third antennal segment. We have recently presented evidence that Hth and Dll induce an unknown signal that acts nonautonomously in specification of the most distal antennal elements (Emerald et al., 2003). Removal of the source of this signal by Antp in the third antennal segment might allow transformation of arista to tarsus.

The 'selector gene' view implies that Antp promotes leg development. Our findings, together with those of earlier reports, seem more compatible with the view that Antp acts as a repressor of antennal identity. We suggest that Antp allows leg identity to emerge in the femur region by preventing coexpression of Hth and Dll. The observation of Stuart et al (1991) that *Tribolium* mutants lacking the entire Hox complex develop antenna-like appendages in all segments supports this view. Now we see little evidence that Antp acts positively to promote leg identity per se.

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